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(54) Preparation of IGF-1 and plasmids for use therein

(57) IGF-1 (insulin-like growth factor 1) is producing by preparing cDNA therefor from mRNA extracted and isolated from human hepatocytes and transforming an E. coli strain with an expression vector wherein said cDNA is inserted as a fusion gene with another gene (e.g. beta-gal), the said vector being a plasmid having an enterokinase recogniton site between IGF-1 gene and the other gene, the resulting fusion protein being cleaved with enterokinase.

Alternatively, the plasmid may have linker DNA between the two genes which contains the codons AAC GAA so as to provide a hydroxylamine cleavage site in the fusion protein. The fusion protein may be reacted with a disulphide compound, such as diethanol disulphide or dithiobisnitrobenzoic acid, in the presence of mercapto-ethanol catalyst to prevent the formation of intramolecular disulphide bonds and expose the recognition site to the cleaving enzyme.

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FIG. 1

ATG	GGA	CCG	GAG	ACG	CTC	TGC	GGG	GCT	GAG
Met	Gly	Pro	Glu	Thr	Leu	Cys	Gly	Ala	Glu
CTG	GTG	GAT	GCT	CTT	CAG	TTC	GTG	TGT	GGA
Leu	Val	Asp	Ala	Leu	Gln	Phe	Val	Cys	Gly
GAC	AGG	GGC	TTT	TAT	TTC	AAC	AAG	CCC	ACA
Asp	Arg	Gly	Phe	Tyr	Phe	Asn	Lys	Pro	Thr
GGG	TAT	GGC	TCC	AGC	AGT	CGG	AGG	GCG	CCT
Gly	Tyr	Gly	Ser	Ser	Ser	Arg	Arg	Ala	Pro
CAG	ACA	GGT	ATG	GTG	GAT	GAG	TGC	TGC	TTC
Gln	Thr	Gly	Ile	Val	Asp	Glu	Cys	Cys	Phe
CGG	AGC	TGT	GAT	CTA	AGG	AGG	CTG	GAG	ATG
Arg	Ser	Cys	Asp	Leu	Arg	Arg	Leu	Glu	Met
TAT	TGC	GCA	CCC	CTC	AAG	CCT	GCC	AAG	TCA
Tyr	Cys	Ala	Pro	Leu	Lys	Pro	Ala	Lys	Ser
GCT	TAG								
Ala	***								

FIG. 2

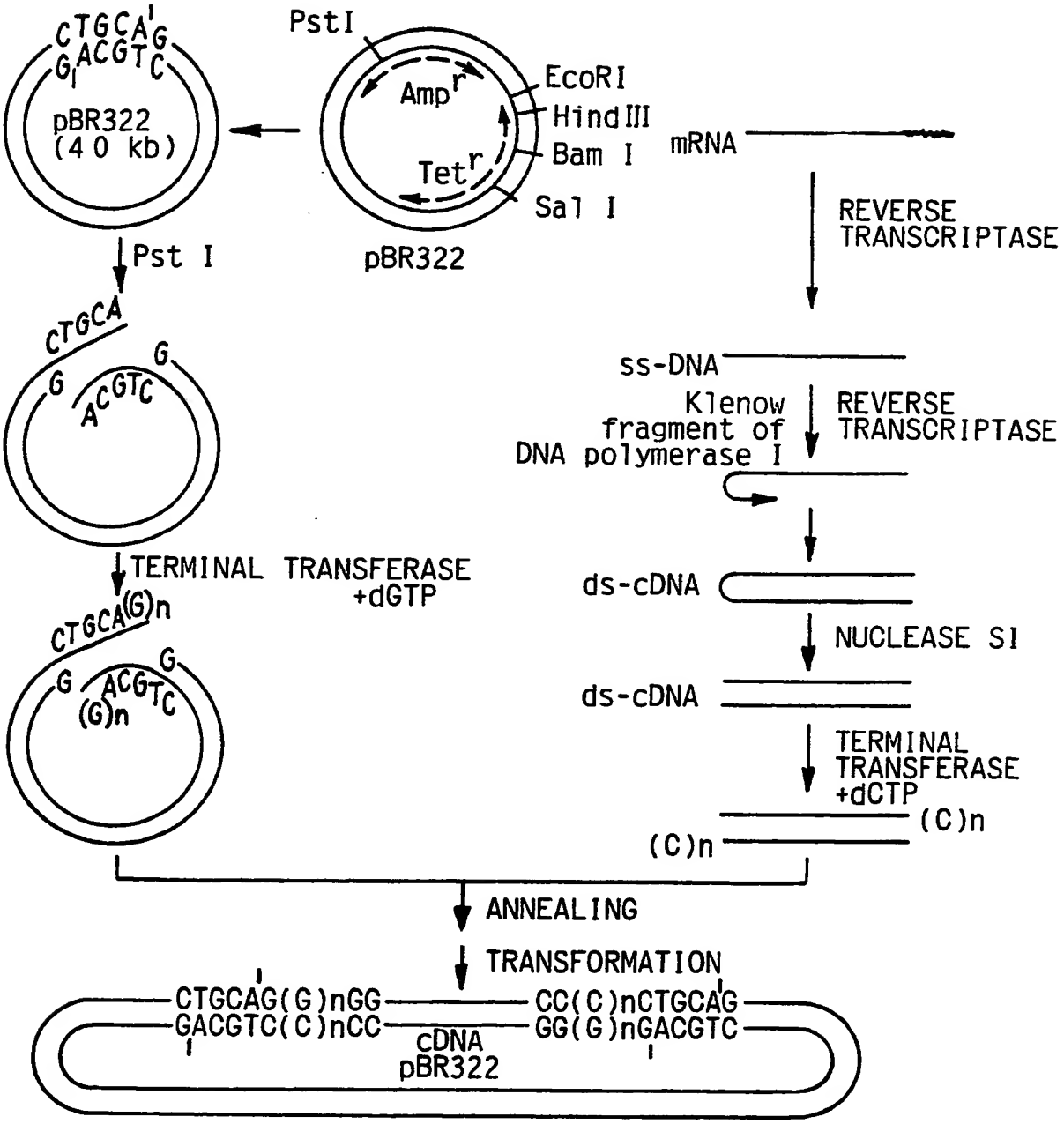


FIG.3

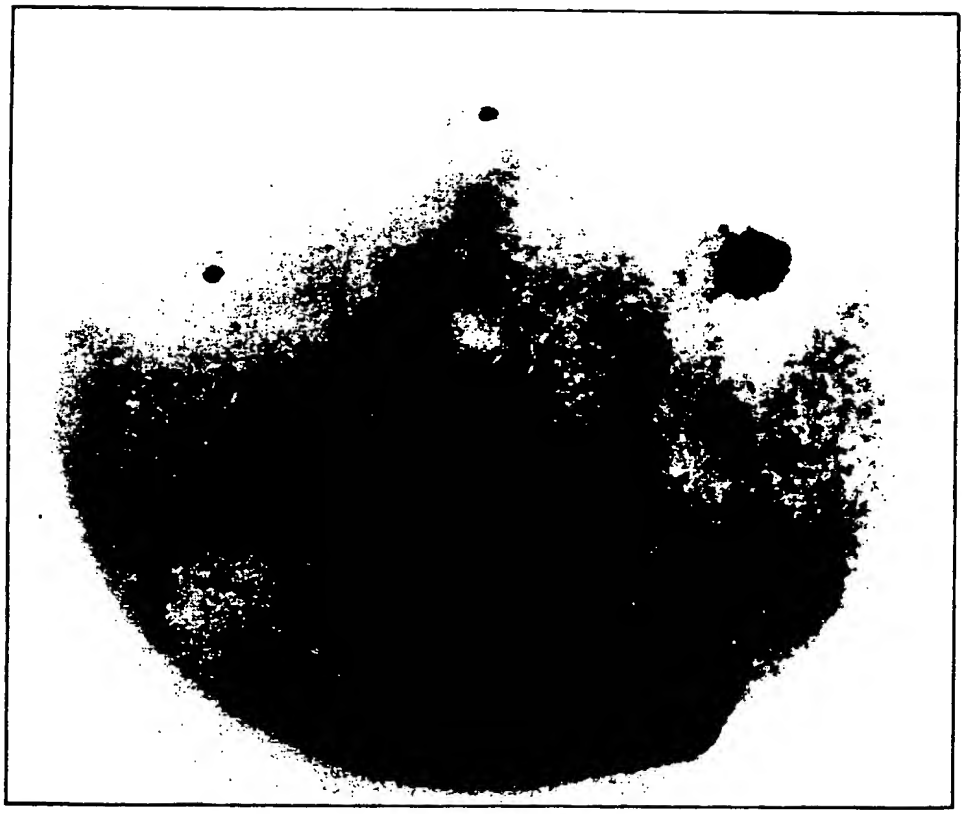
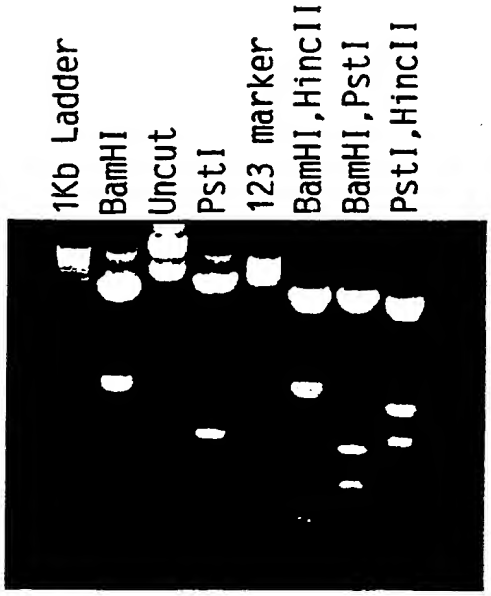


FIG.4



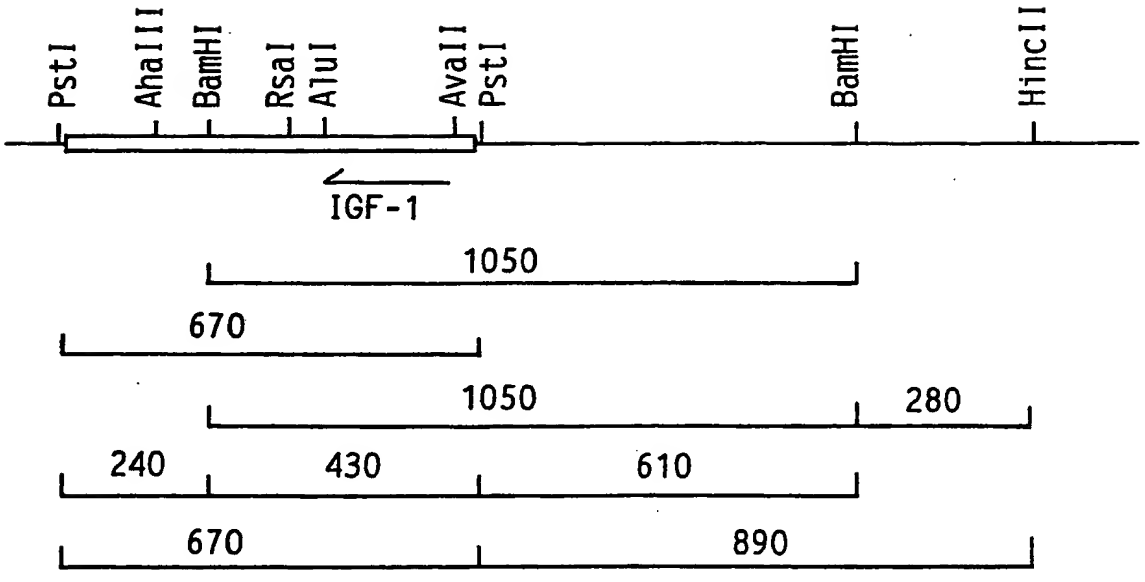


FIG.5

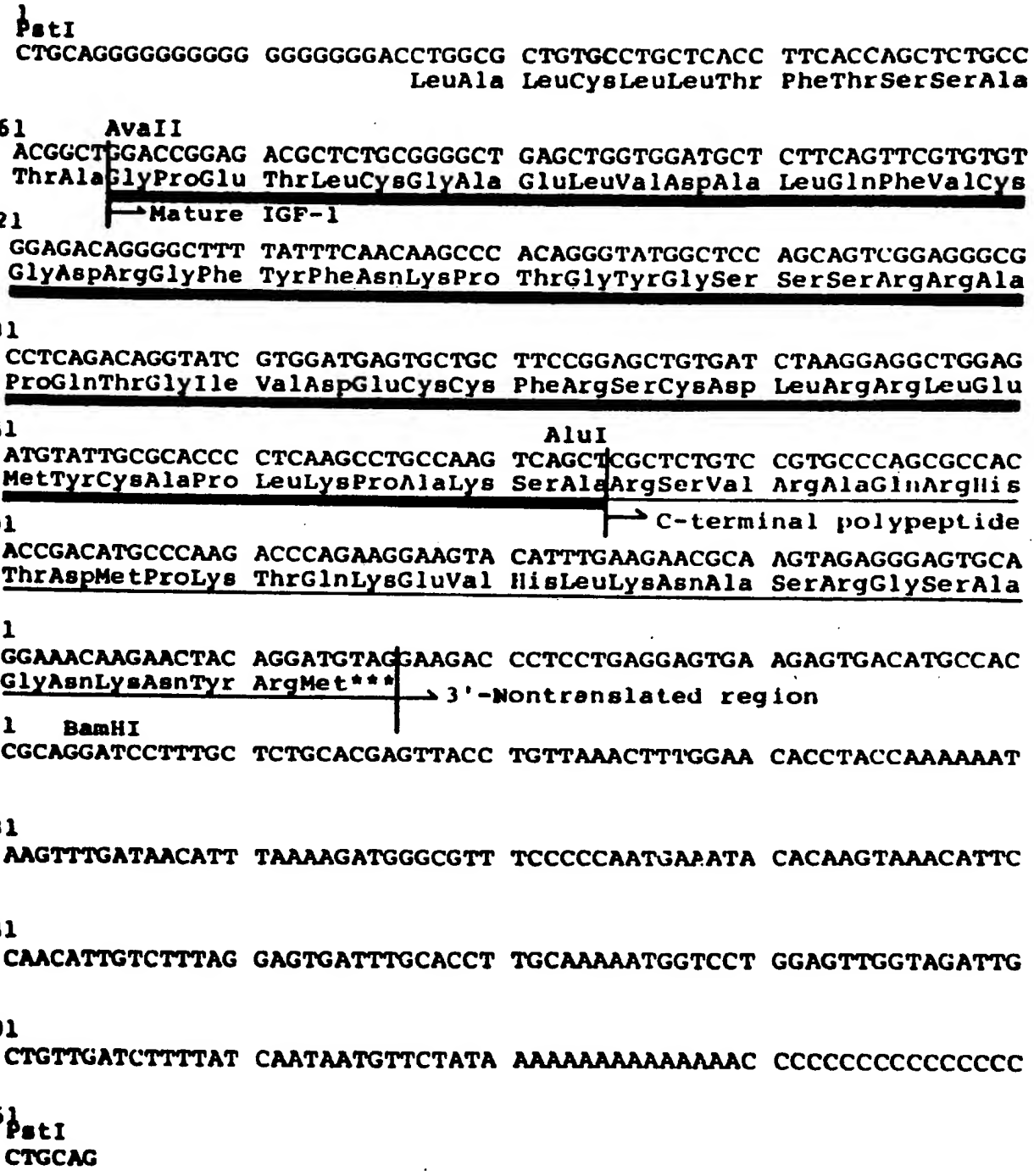


FIG.6 6/18

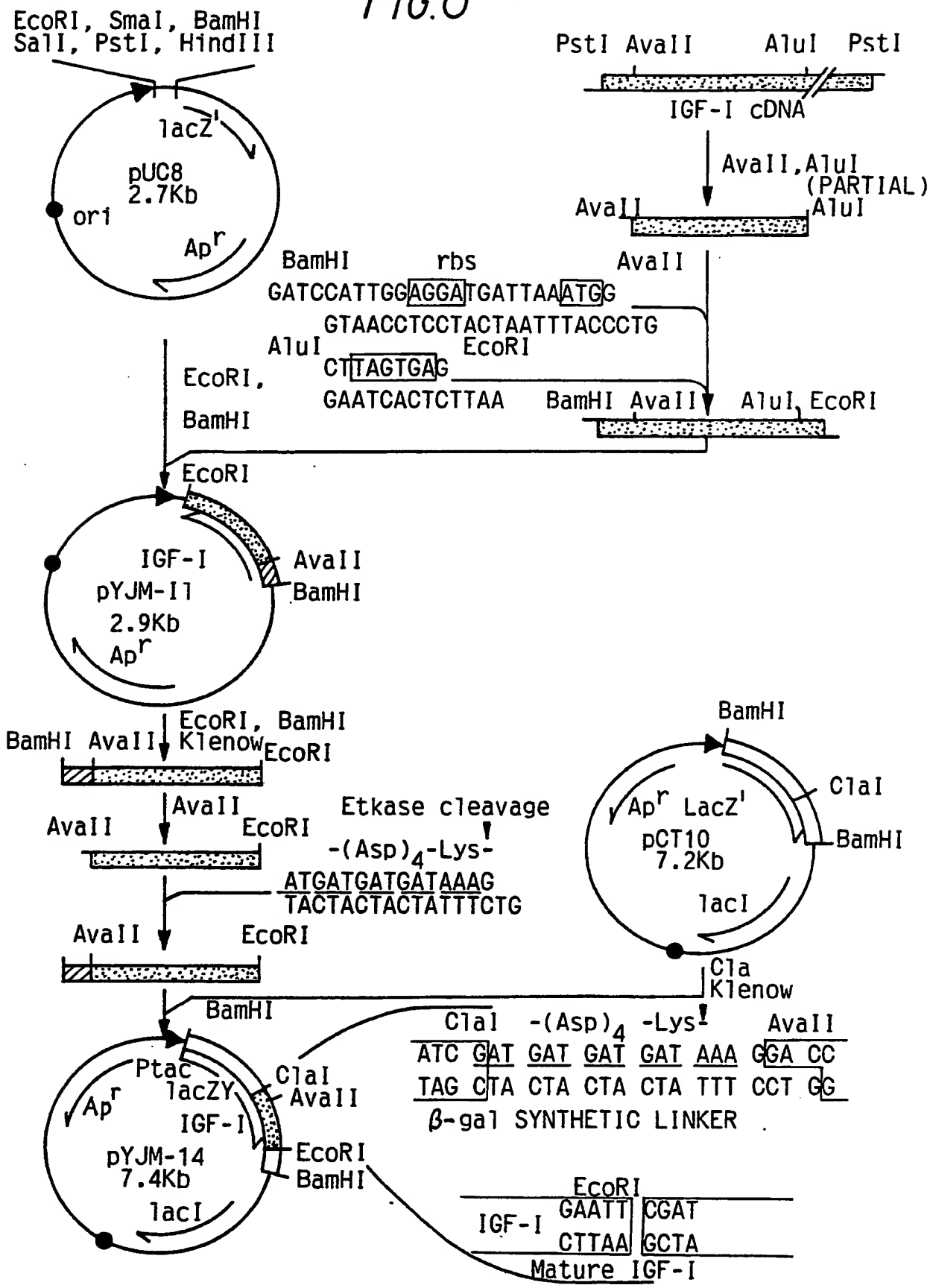


FIG. 7

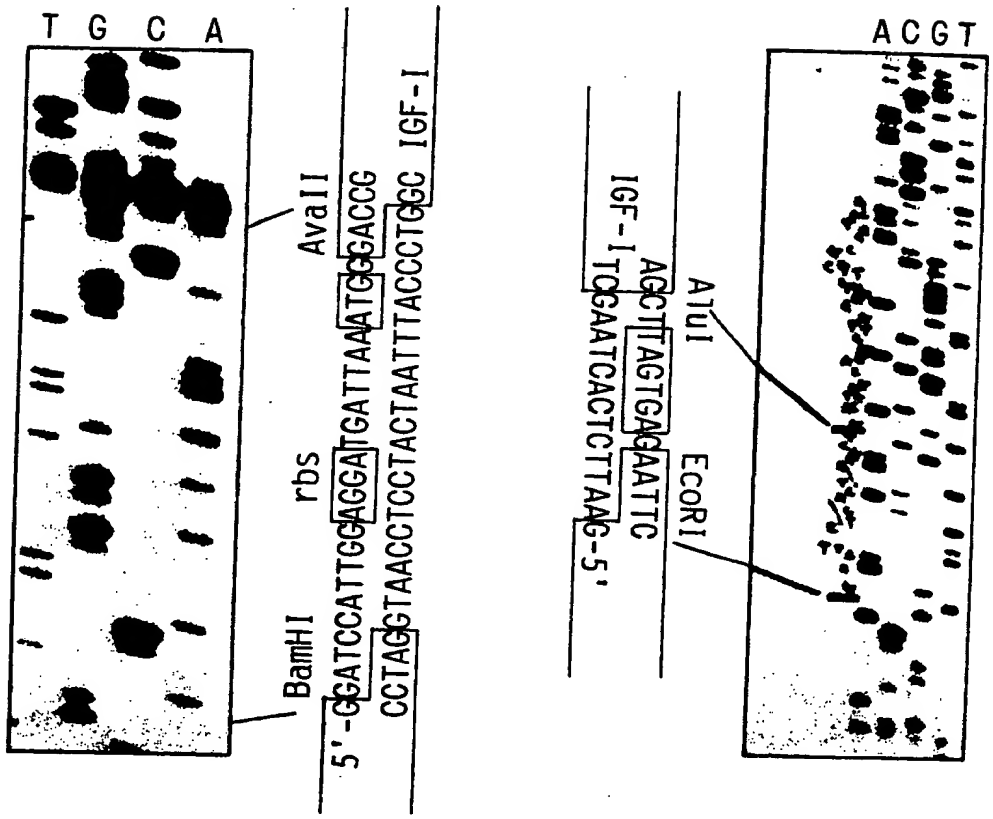


FIG.8

FIG. 9

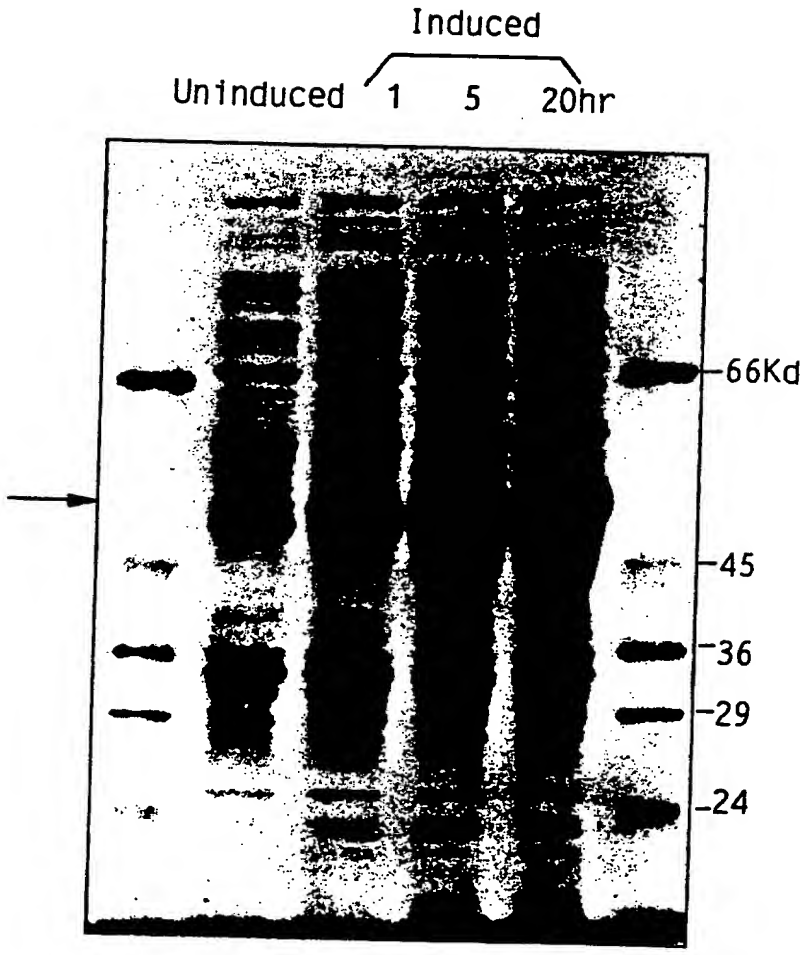


FIG. 10

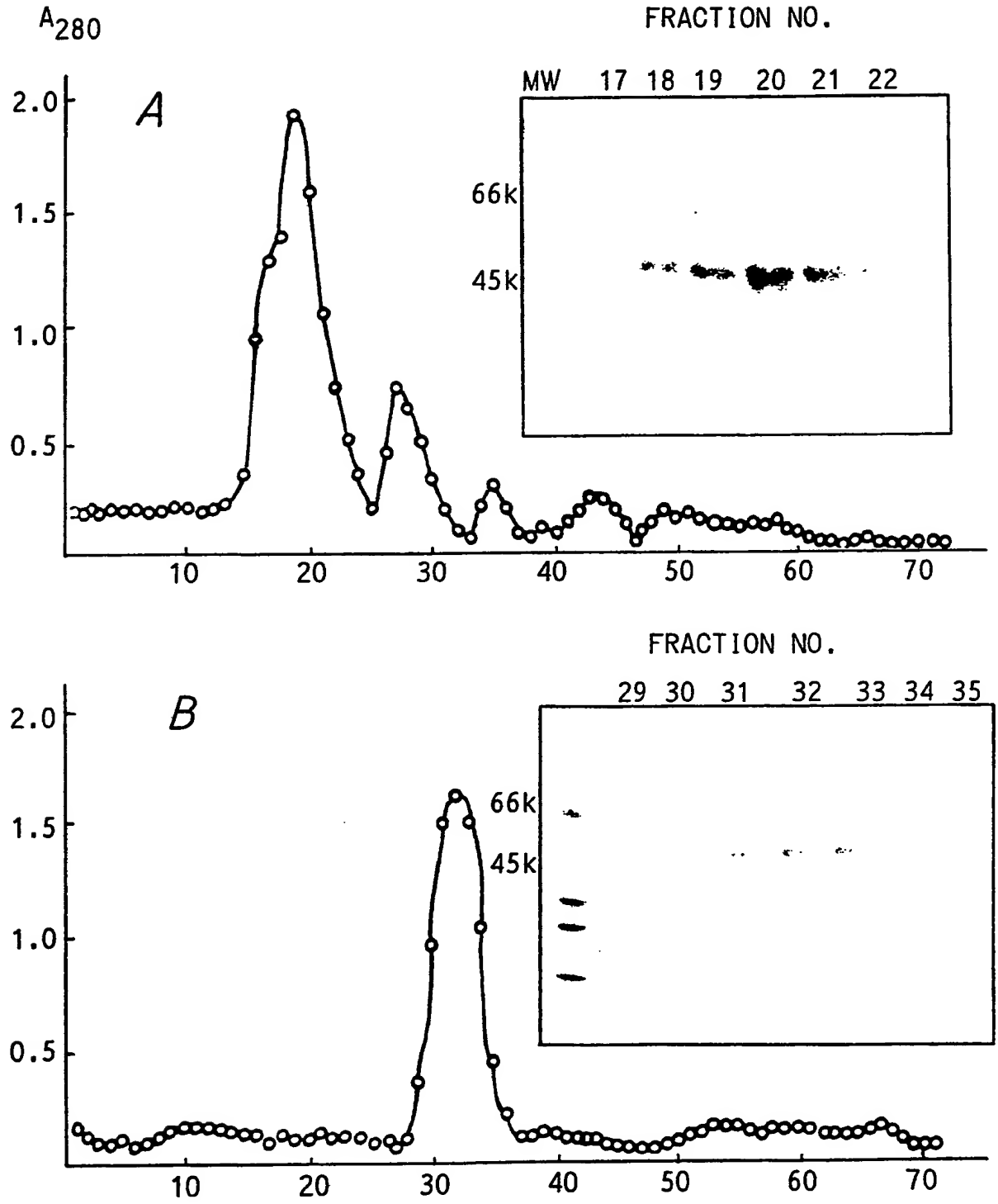


FIG. 11

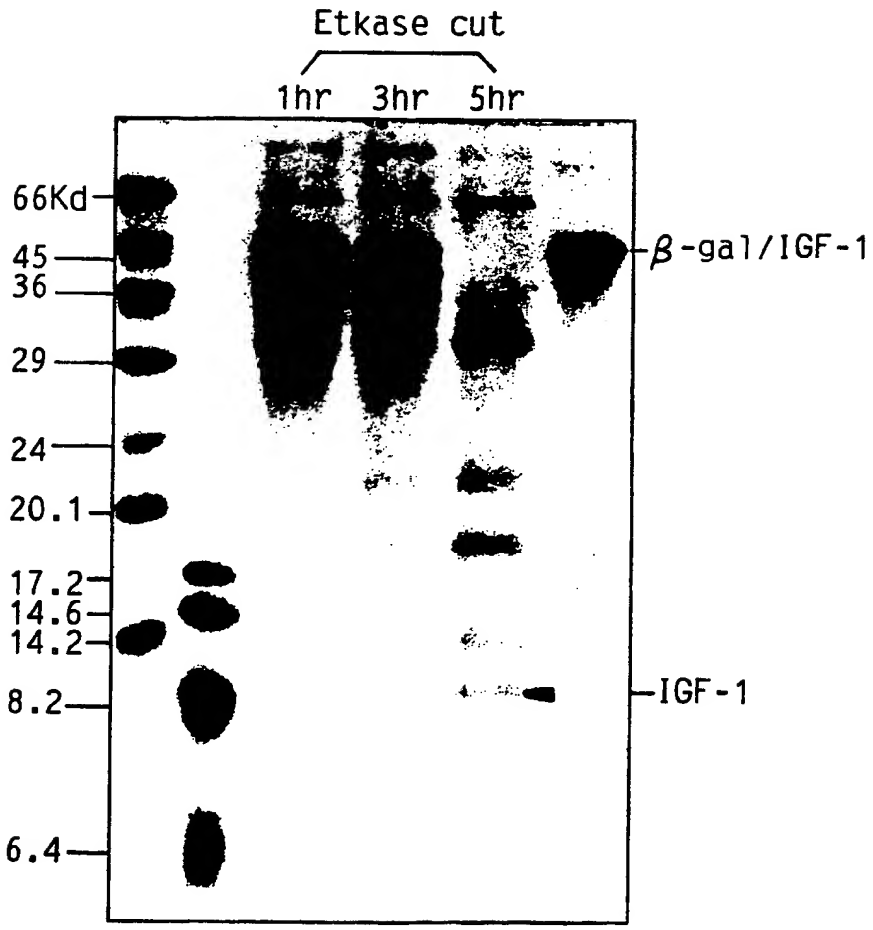


FIG. 12

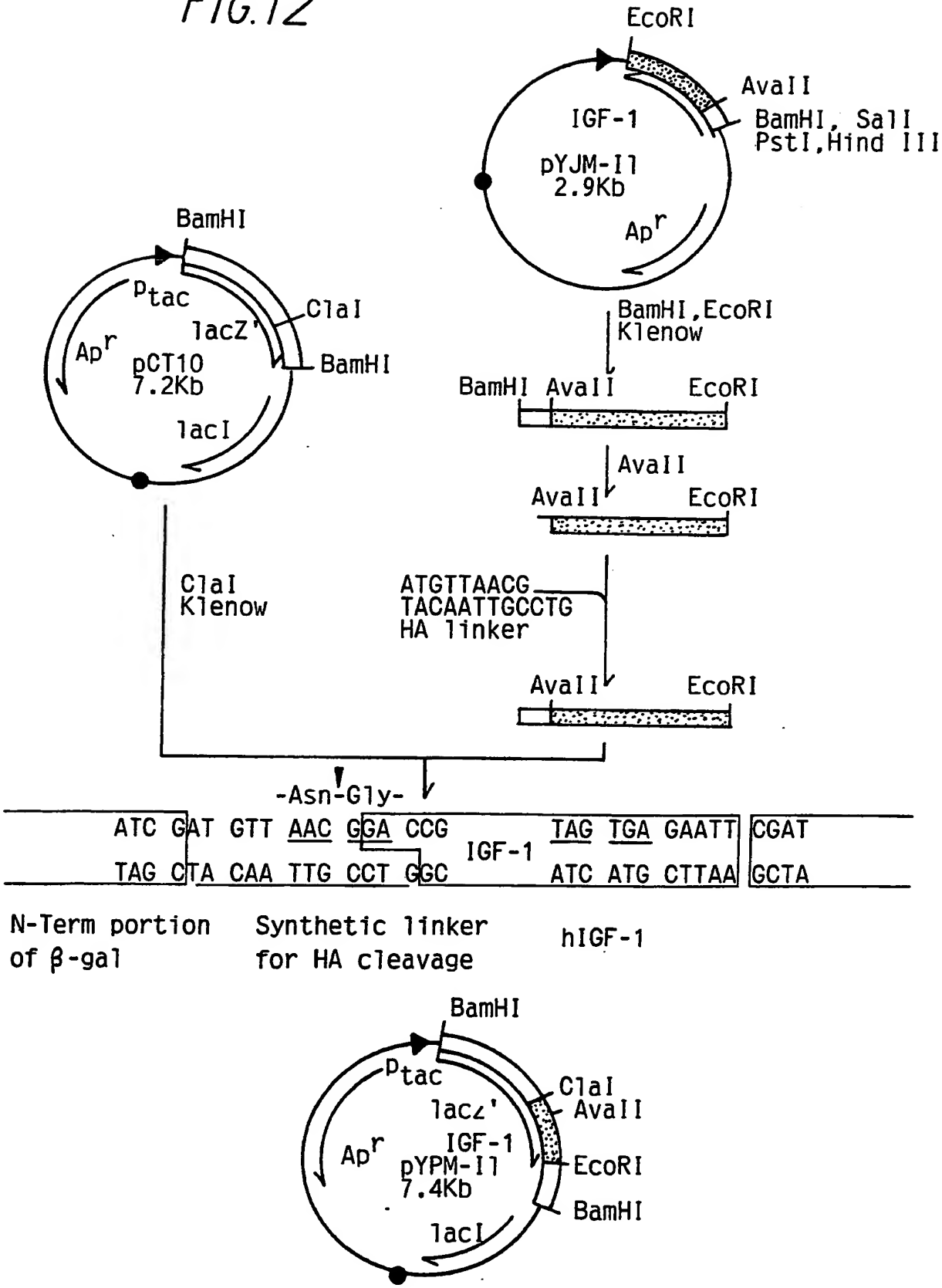
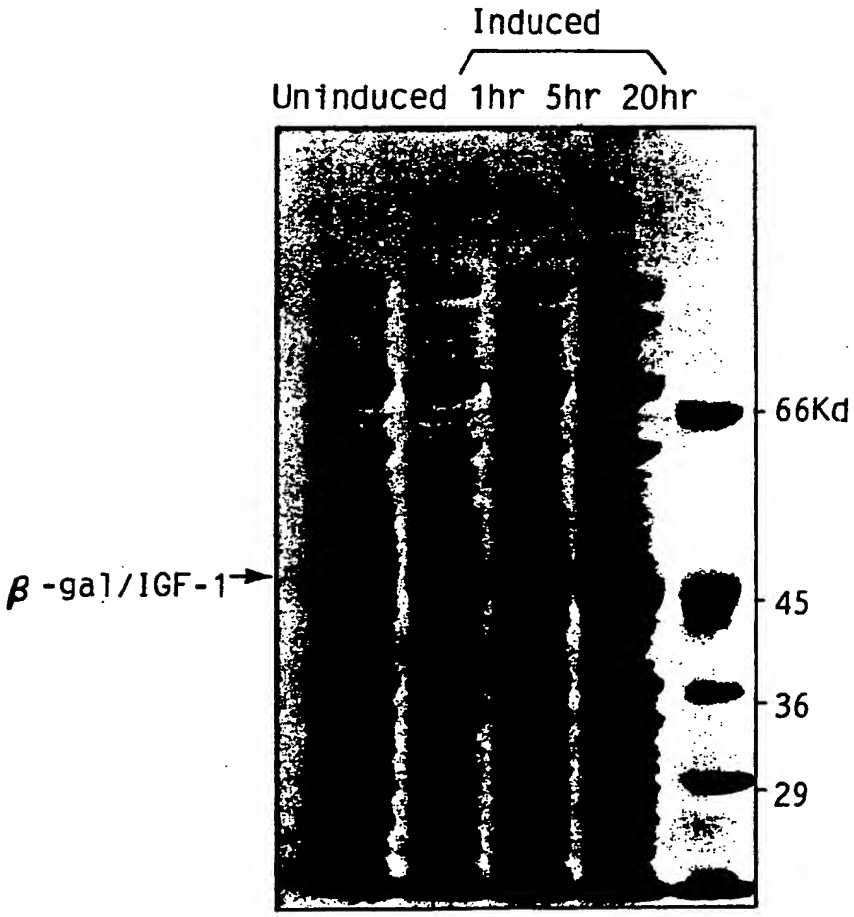


FIG. 13



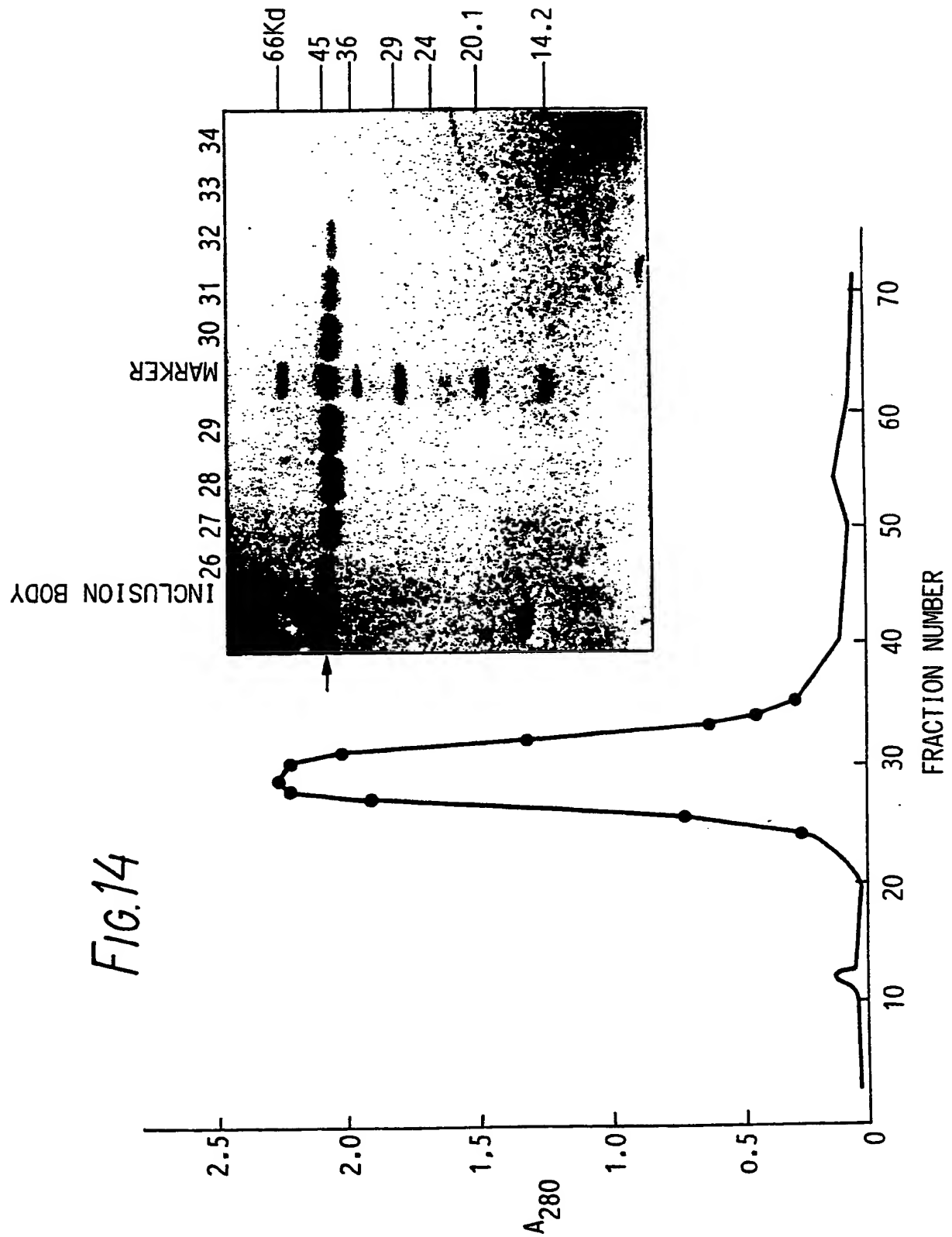


FIG.14

FIG. 15

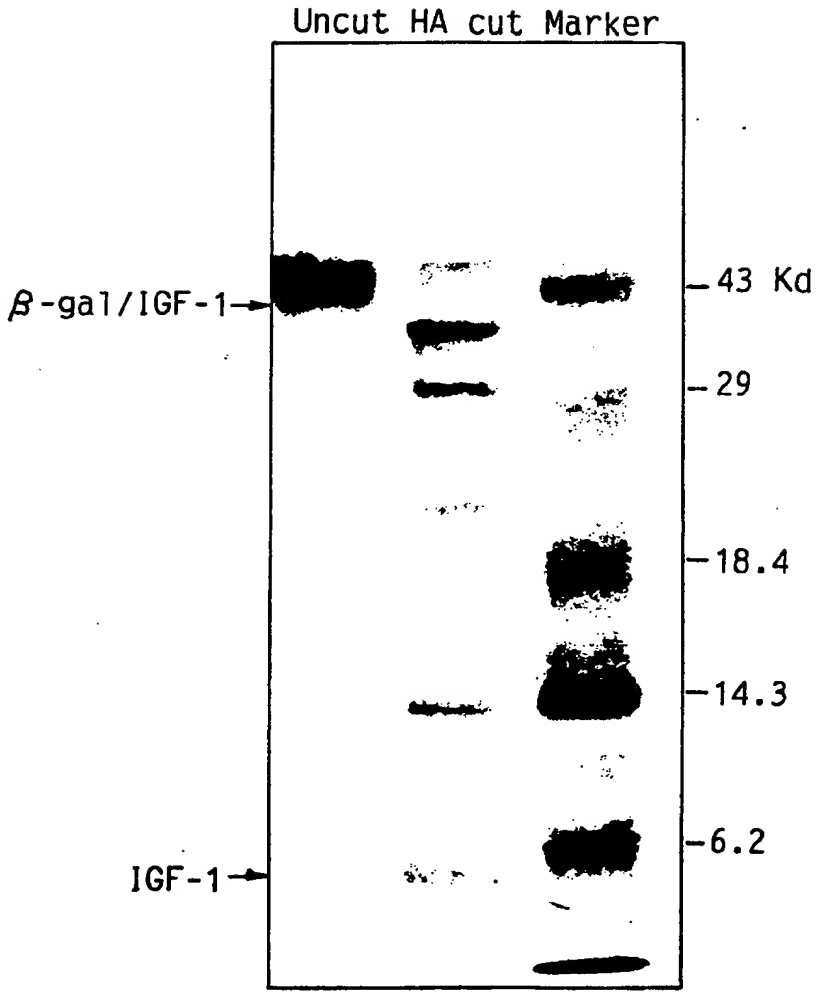
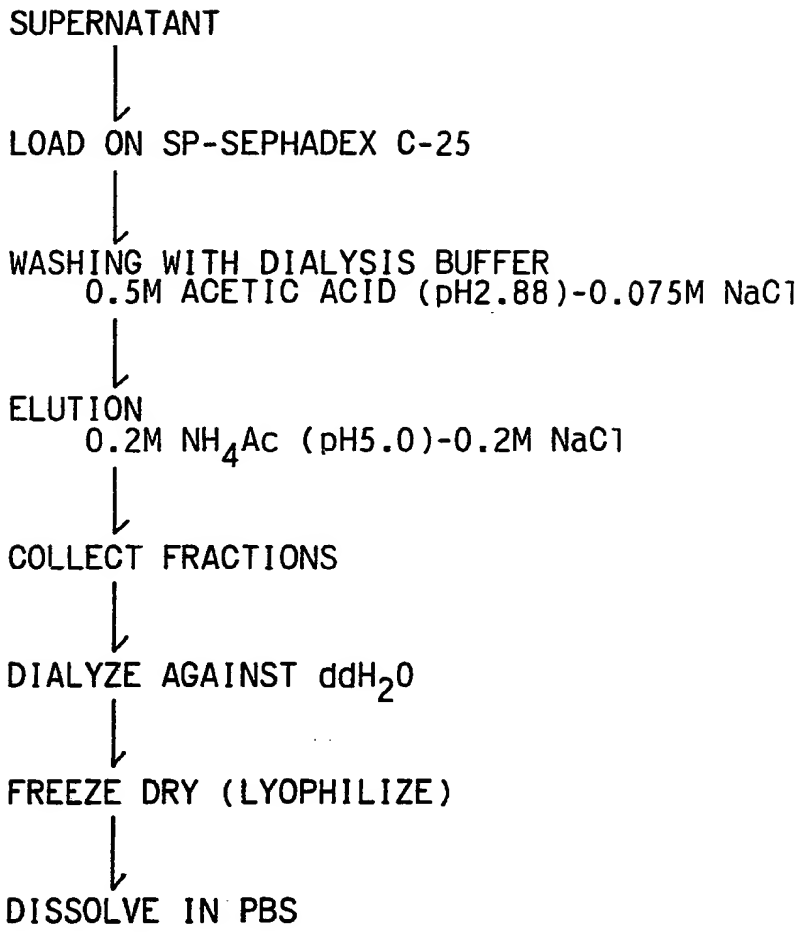


FIG. 16



SP-SEPHADEX C-25 COLUMN CHROMATOGRAPHY OF IGF-I

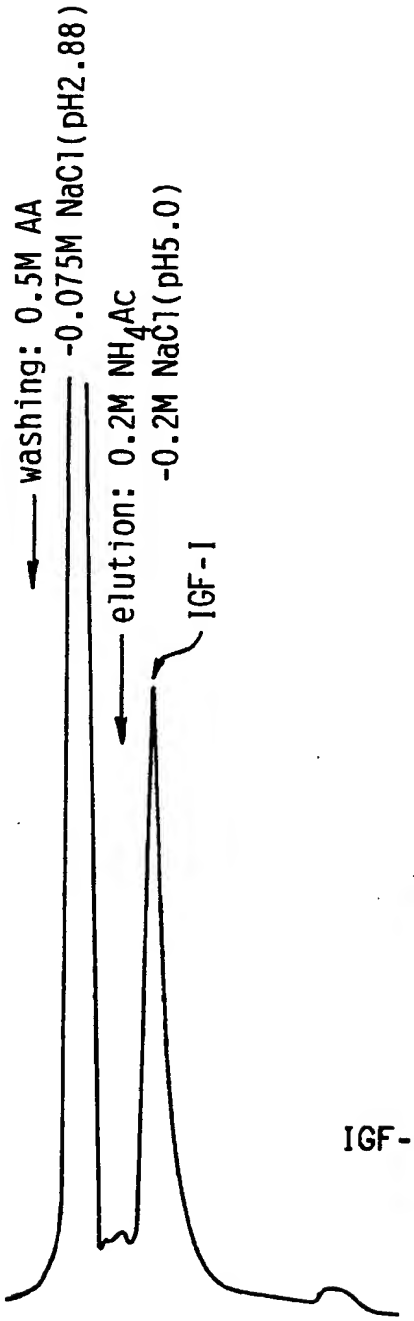


FIG. 16 cont.

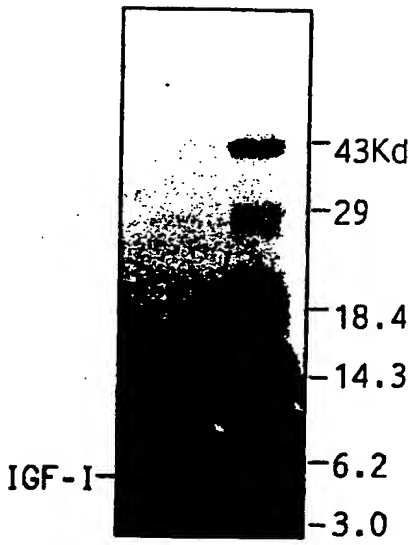
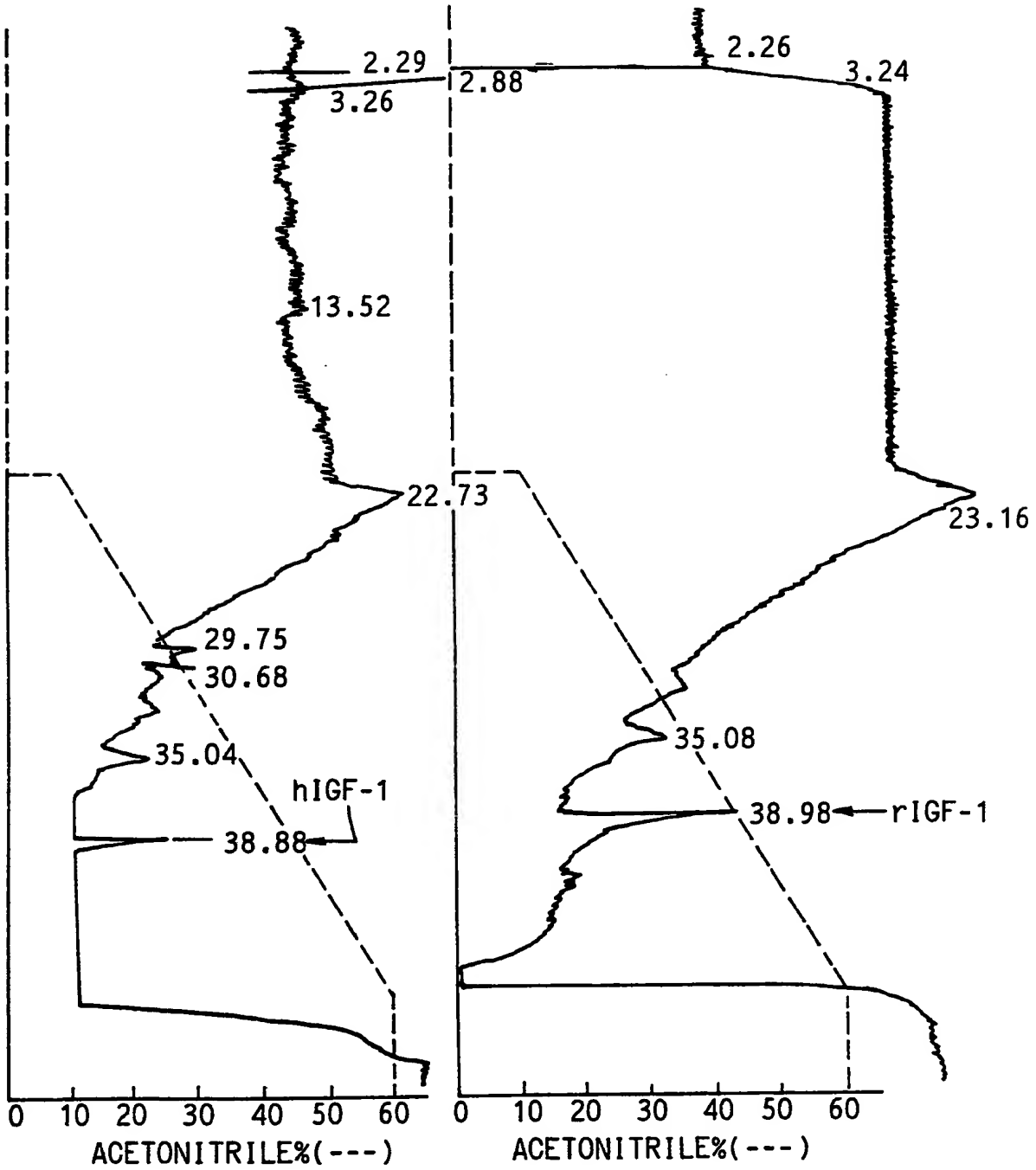


FIG.17



PREPARATION OF A NOVEL PLASMID AND A METHOD FOR PRODUCING IGF-1 BY CULTURING MICROORGANISMS CONTAINING SAID PLASMID

This invention relates to the preparation of a novel plasmid and a method for producing IGF-1 by culturing microorganisms containing said plasmid.

2. BRIEF DESCRIPTION OF DRAWINGS

Figure 1 depicts the base sequence of gene encoding IGF-1 as well as the amino acid sequence translated therefrom.

Figure 2 depicts the preparation of a cDNA library using poly A messenger RNA derived from liver cell.

Figure 3 represents a colony hybridization for the isolation of IGF-1 cDNA using a 15-mer probe labelled with radioisotope.

Figure 4 represents the restriction enzyme map of the gene encoding IGF-1.

Figure 5 represents the DNA base sequence of the IGF-1 gene and its surrounding base sequence.

Figure 6 is a systematic diagram showing the procedure for the construction of the IGF-1 expression vector pYJM-I4.

Figure 7 represents the assay for determining the DNA base sequences at the 5'- and 3'-end of the IGF-1 gene in the IGF-1 expression vector pYJM-I1.

Figure 8 represents an electron micrograph of a fusion protein of β-galactosidase and IGF-1 which has been expressed in E.coli and accumulated as inclusion bodies.

Figure 9 is a photograph of SDS-polyacrylamide gel electrophoresis of IGF-1 produced by using the expression vector pYJM-I4 which is then identified with SDS-PAGE.

Figure 10 explains the purification of the inclusion bodies:

(A) Profile and electrophoresis photograph of the material passing through the first Sephacryl S 200 column.

(B) Profile and electrophoresis photograph of the material passing through the second Sephacryl S 200 column.

Figure 11 represents an electrophoresis photograph showing a fusion protein of IGF-1 and β-galactosidase, which has been hydrolyzed with enterokinase and then identified by using SDS-PAGE.

Figure 12 is a systematic diagram showing the procedure for constructing the IGF-1 expression vector pYPM-I1.

Figure 13 is an electrophoresis photograph of IGF-1 produced by utilizing the vector pYPM-I1 which is then identified with SDS-PAGE.

Figure 14 shows the results of the purification of the inclusion bodies that pass through a Sephacryl S 200 column and a photograph of their appearance on SDS-PAGE.

Figure 15 is a photograph showing the fusion protein of IGF-1 and β - galactosidase which has been cleaved with hydroxylamine and then identified by using SDS-PAGE.

Figure 16 represents the results of the purification of IGF-1 using SP-Sephadex C-25 column and a photograph of its presentation on SDS-PAGE.

Figure 17 is a graph showing the results of measuring the purity of the HPLC-isolated IGF-1.

DETAILED DESCRIPTION OF THE INVENTION

IGF-1 (insulin like growth Factor-1), which is produced in the liver under the control of human growth hormone and then released into the blood has a molecular weight of 7,649 and is composed of about 70 amino acids.

The amino acid composition of IGF-1 has about 43% similarity to the A-chain of insulin. Thus, IGF-1 is capable of binding to the insulin receptor as well as the IGF-1 receptor. It has been known that IGF-1 functions as insulin in living bodies and stimulates the growth of cells.

IGF-1 is a type of somatomedin. In the living body, three kinds of somatomedins (A,B,C) are present, among which somatomedin C is designated as IGF-1.

The receptors for IGF-1 are present on adipose tissue, lymphocytes, bone, and placental membrane, etc., as well as hepatocyte. According to the experimental results, it has been found that these IGF-1 receptors are different from insulin receptors.

The binding of somatomedin C to the IGF-1 receptor stimulates the second messenger, which has not yet been identified and thus, results in the stimulation of mitosis. In vivo, IGF-1 which has been secreted from liver into blood, is combined with a carrier protein in blood, and thus, circulates in an inactive form. Then, IGF-1 is separated from the carrier protein depending on the nutritive state and physiological changes in the human body, and then is combined with the receptor present on somatic cells, thereby inducing its mitogenic effect.

Biological activities induced by IGF-1 can be classified into a short-term effects and a long-term effects.

The short-term effect is mainly the same phenomenon as the effect resulting from the binding of insulin to the insulin receptor present in adipose tissue or heart muscle cell upon which insulin exerts its action. That is, in the same manner as insulin, IGF-1 stimulates the transportation of glucose and the synthesis of fat from glucose by oxidation of the absorbed glucose.

Such an IGF-1 activity results from the binding of IGF-1 to the insulin receptor. This has been proved by the experiments showing that when the insulin receptor present in adipose tissue is removed by trypsin treatment, the activity of IGF-1 disappears [King et al., J. of Clinical Invest. 66:130 (1980)].

In contrast with the short-term activity of IGF-1 showing the same effect as insulin, the long-term activity of IGF-1 results in the stimulation of replication, growth and differentiation of various tissue cells in human body, contrary to insulin activity. It has been disclosed that when cartilage cell from chicken or rat is treated with IGF-1, there is a rapid increase in the absorption of sulfate groups as well as in the synthesis of ribonucleic acids (RNA) involved in the growth of cartilage [steiner, Th et al. Calcified Tissue International 35 : 578 (1983)].

In addition, since the treatment with IGF-1 increases the concentration of acetylcholinesterase and creatine kinase, which essentially present in muscle cells, within somatic cell wall and cells in stomach of chicken, it is evident that IGF-1 acts upon the differentiation of these undifferentiated cells into muscle cells.

Further, in the year of 1982, Kurzz and Baner reported their results comparing the effects of insulin and IGF-1 on erythrocytes isolated from rat liver and bone marrow. They showed that the IGF-1 treatment had a considerably greater effect on cell replication in comparison with the insulin treatment.

The long-term effect of IGF-1 has been more clearly demonstrated according to the results of in vivo studies rather than in vitro studies. Specifically, mice whose pituitary gland had been removed were injected with growth hormone, IGF-1 or insulin alone or in various combinations and then, the effects thereof were determined. As a result, in mice who had received only IGF-1 without growth hormone, the diameter of the hard bone tissue became great, and the content of sulfur and thymidine increased in the cartilage, and there was a prominent weight gain, in comparison with mice who have received the other treatments [Scohoenle et al., Nature 296 : 252 (1982)].

Since IGF-1 exhibits such a strong mitogenic effect, it may be very useful as a growth stimulating agent.

The present invention relates to the production of large quantities of IGF-1 which comprises isolating the IGF-1 gene from human hepatocytes, inserting the isolated IGF-1 gene into a unique expression vector and then introducing this vector into a microorganism. The present invention further relates to the use of IGF-1, which is expressed and isolated as above, for controlling the blood glucose level in diabetic patients, who cannot be treated with insulin, for local treatment of bone fracture and for treating congenital or acquired growth failure patients.

The method for expression and purification of IGF-1 according to the present invention has been developed as a novel method which overcomes many of the technical problems involved in the prior art.

When it is very difficult to express a given gene directly, the prior art has used a method wherein the gene product is expressed in the form of a fusion protein with the other protein and then the fusion protein is cleaved using a chemical reagent (CNBr). However, such a method is disadvantageous in the production of IGF-1 because the use of IGF-1 in the human body is very difficult even after its purification, due to the toxicity of chemical reagent (CNBr) itself.

Thus, the present invention has selected a more accurate method wherein the fusion protein is precisely cleaved by using an enzymatic method and hydroxylamine, rather than CNBr used in the prior art, and then the desired protein is purified.

The enzyme selected in the present invention is enterokinase. Thus, the present invention uses a method wherein the nucleotides CAT-CAT-CAT-CAT-AAA-, which encode five amino acids (Asp-Asp-Asp-Asp-Lys-) upon which enterokinase acts, are synthesized and then inserted into the cleaved site of the fused gene, and the obtained gene is expressed and then the desired IGF-1 is separated by cleaving the obtained product with enterokinase. This is a novel method which has never been used in the prior art.

In addition, the present invention uses the protein R-radical substitution method for cleaving the inclusion bodies of the fusion protein expressed using the above novel method at the desired position. Said protein R-radical substitution method is also a novel method which has never been used in the prior art.

The fusion protein expressed in E. coli is present in the form of inclusion bodies in which many of the cystein residues in the fusion proteins are randomly combined with each other to form disulfide bonds. Therefore, the use of a strong reducing agent such as DTT or β-mercaptoethanol is required to denature and solubilize the fusion protein. However, when a strong reducing agent is used to cleave the disufide bonds within the fusion protein the activity of enterokinase which cuts off the fusion protein is destroyed. Furthermore, in case of the reduction of a sulfhydryl group (-SH) of cystein using a reducing agent such as β-mercaptoethanol, the resultant is very unstable. Specifically, in this case, when β-mercaptoethanol which is not combined, is removed in the presence of oxygen, the re-oxidation occurs readily to reform disulfide bond and the fusion protein reprecipitates as an inclusion body.

Due to the numerous above-mentioned problems, before the present invention, IGF-1 could only be produced in its dissolved state with a very low efficiency in E. Coli. Thus, the present invention utilizes a novel method, as explained below, which overcomes such problems.

First, disulfide compounds (diethanol disulfide, dithiobisnitrobenzoic acid) which combine with the sulfhydryl group of cystein and are not readily oxidized at the normal temperature, are reacted with the fusion protein in the presence of β-mercaptoethanol catalyst to prevent the formation of disulfide bonds between -SH groups present in a fusion protein. In such a manner, a portion of the fusion protein to be cleaved with enzyme is sufficiently exposed to enterokinase enzyme. After the fusion protein is cleaved with enterokinase as mentioned above, IGF-1 is separated and isolated therefrom.

The removal of disulfide compounds from IGF-1 combined with disulfide compounds at the sulfhydryl group of cystein can be achieved by gradually dialyzing out the disulfide compound with β-mercaptoethanol catalyst having the lower concentration than that of the combining condition. According to this, the disulfide compounds are removed and IGF-1 is re-activated and thus can be isolated.

As mentioned above, the present invention utilizes a novel method for cleaving the expressed fusion gene product using a specific enzyme, and also, after the mass expression of IGF-1 fusion protein, utilizes a method wherein said fusion protein is solubilizes using the R-group substitution method before it is cleaved with the enzyme.

In addition to the method wherein the R-group substitution is carried out and then the fusion protein is cleaved with enzyme (enterokinase), the present invention also provides a method for producing pure IGF-1 by utilizing hydroxylamine. Hydroxylamine can specifically cleave the bond between Asn-Gly residues in the protein when used at PH 9. By utilizing this principle, ATGTTAACG and TACAATTGCCTG are synthesized and then used as a linker, thereby forming the AAC, GGA codons encoding Asn-Gly between β-gal and IGF-1. Therefore, if the β-gal / IGF-1 fusion protein is produced and then is cleaved with hydroxylamine, hydroxylamine cleaves the portion between Asn-Gly within the fusion protein and thus, IGF-1 having glucine as its first amino acid can be obtained in a pure state.

The novel method of the present invention as mentioned above can produce IGF-1 in a considerably high yield using a simple procedure, in comparison with the prior methods.

In order to produce a certain protein, first, mRNA must be obtained from cells capable of producing the desired protein. The mRNA thus obtained are converted into cDNA by means of reverse transcriptase, to which (dC)n tail is attached. The (dC)n-tailed cDNA is annealed with (dG)n-tailed vector (pBR 322) to produce the recombinant vector which is then introduced into a suitable host strain for transformation.

Thus transformed strain contains various cDNAs corresponding to various mRNAs. Thus, a population of transformed strain produced as mentioned above is called a cDNA-library. In order to screen and isolate the strain containing the desired gene from all of the transformed strains produced above, the colony hybridization method is utilized [Maniatis, T., et al. 1982, "Molecular Cloning", pp. 312-319]. For this method, the base sequence corresponding to five amino acids present in the previously known peptide sequence is synthesized using a DNA synthesizer and then labelled with ^{32}P, so that it can be used as a probe. For the expression of IGF-1 coding gene thus produced, an efficient expression vector must be utilized. Although various vectors can be used for expressing a gene from a higher animal in E. Coli, the present invention uses a vector containing the tac promoter (See : Figure 6).

As IGF-1 cDNA clone which has been isolated by colony hybridization is treated with various restriction endonucleases to identify the desired gene (See : Figure 3). As can be seen from Figure 5, IGF-1 cDNA is inserted as a 680 bp fragment into between the two Pst I restriction enzyme sites of a vector derived from pBR 322. Within the 680 base pair fragment containing IGF-1 cDNA, the portion between the Ava II and Alu I restriction enzyme sites, which is identified by the bracket in Figure 5, is used to express IGF-1, which has been further processed post-translationally in E. Coli.

For construction of the expression vector pYJM-I4 for expressing IGF-1 in E. Coli, the following genetic engineering procedures are carried out (See : Figure 6). IGF-1 cDNA is treated with Ava II restriction enzyme and then slightly treated with Alu I enzyme to obtain the 206 bp Ava II-Alu I DNA segment which is then separated by gel electrophoresis.

The four kinds of synthetic oligonucleotides, i.e., GATCTGGAT-GATTAAATGG (MG-6), GTCCCATTTAATCATCCTCCAATG (MG-7), CTTAGAG (MG-8) and AATTCTCACTAAG (MG-9) are mixed and ligated. The obtained ligation product is inserted into the portion between the BamHI and EcoRI recognition sites of plasmid pUC 8 to prepare plasmid pYJM-I1 (Fig. 6(A), Deposit Nos. KCTC 8247p and KCTC 0004BP). The plasmid pYJM-11 thus constructed contains a gene encoding 70 amino acids of IGF-1, which are formed via post-translational processing, between the recognition sites for two restriction enzymes BamHI and EcoRI. Thus, in order to fuse the gene for IGF-1 with β-galactosidase and then to express the fusion protein, the gene for IGF-1 is inserted into the expression vector pCT 10 (See : Figure 6(B) for the fusion with β-galactosidase, according to the following procedure, to construct the expression vector pYJM-I4 for expressing IGF-1 in E. Coli. The plasmid pYJM-I1 is treated with restriction enzymes BamHI and EcoRI to separate IGF-1 DNA which is filled in at both ends by using DNA polymerase Klenow fragment enzyme of E. Coli. The resulting fragments are treated with Ava II restriction enzyme to separate the 220 bp IGF-1 DNA fragment by gel electrophoresis, as can be seen from Figure 6. Then, the 220 bp IGF-1 DNA fragment is mixed and ligated with two kinds of oligonucleotides, ATGATGATGATAAAG (MG-24) and GTCCTTTATCATC-ATCAT (MG-25). The ligated 235 bp DNA fragment is inserted into pCT 10 at Cla I site, which has been treated with Klenow enzyme, to construct plasmid pYJM-I4 (See : Figure 6(B), Deposit Number KCTC 8428p and KCTC 0005BP).

As can be seen from figure 6, the plasmid pYJM-I4 comprises the antibiotic ampicillin-resistant gene (Ap^r), a colE1 replication site (ori.), and a lac I gene (lac I) producing lac repressor. In the plasmid pYJM-I4, the DNA sequence encoding fusion protein of β-galactosidase and IGF-1 is linked with the tac promoter, which is a strong promoter of E. coli, thereby producing a great quantity of the fusion protein of β-galactosidase and IGF-1. In addition, since the DNA base sequence corresponding to amino acids sequence $(Asp)_4$-Lys, which is the enzyme enterokinase recognition site, is present in the portion of the DNA sequence linking β-galactosidase with IGF-1 protein, the treatment of the expressed β-gal/IGF-1 fusion protein with enterokinase permits the isolation of only the IGF-1 protein.

100ml of M9 + CA minimal medium containing 100 μg/ml of ampicillin is inoculated with 5ml of seed culture, which has been prepared by culturing overnight E. coli strain JM 109 containing the expression vector pYJM-I4 in LB+Ap (100 μg/ml) medium, and then inoculated medium is cultured for 3 hours at 37 °C. To the culture, 2mM of IPTG (iso-propyl-β-D-thio-galactoside) is added to induce the tac promoter to produce the β-galactosidase/IGF-1 fusion protein in E. coli. Each 1.5ml of cultures thus obtained is harvested, a portion of which is identified by SDS-PAGE. As a result, in comparison with the state before the induction a strong protein band is observed at the position of molecular weight of about 50,000 daltons with the time lapse of 1 hour, 4 hours and 5 hours. From this result, it can be seen that the fusion protein of β-galacto-sidase and IGF-1 is expressed under the control of the tac promoter of E. coli (see : Figure 9).

In addition, it can also be seen that according to the above procedure, the desired protein is efficiently produced at levels above 20 to 30% of all the E. coli proteins. From an electron micrograph, it has been noted that the produced fusion proteins of IGF-1 and β-galactosidase are accumulated as inclusion bodies in E. coli cells (see : Figure 8).

The fusion protein of IGF-1 and β-galactosidase is isolated and hydrolyzed with enterokinase and then identified with SDS-PAGE to obtain an electrophoresis photograph as shown in Figure 11. As a result, an isolated IGF-1 having the molecular weight of 8,000 daltons is obtained.

The second method utilizes hydroxylamine to cleave the fusion protein. Thus, the expression vector pYPM-I1 (Deposit Nos. KCTC 8465 and KCTC 0006BP)producing the fusion protein of IGF-1 and β-galactosidase, which can be cleaved with hydroxylamine to isolate IGF-1, is constructed as follows.

Plasmid pYJM-I1 is treated with BamHI and EcoRI to isolate the IGF-1 DNA fragment which is then treated with E. coli Klenow fragment enzyme followed by Ava II treatment to isolate the 220bp IGF-1 DNA fragment, as shown in Figure 12. Then, synthetic oligonucleotides ATGTTAACG and GTCCGTTAACAT are mixed and ligated with the 220bp DNA fragment obtained above. The resulting ligation product is inserted into pCT 10 at Cla I site treated with Klenow fragment to propare plasmid pYPM-I1. Since the fusion protein produced by plasmid pYPM-I1 contains an Asn-Gly residue between β-galactosidase and IGF-1, the fusion protein can be treated with hydroxylamine to isolate IGF-1 only.

In the transformed E. coli strain JM 109 containing the expression vector pYPM-I1, the production of the fusion protein of β-galacatosidase and IGF-1 is induced by adding IPTG to the culture. The resulting protein is then identified with SDS-PAGE (Figure 13).

According to this method, an about 45,000 dalton protein can be identified while it is not identified before induction. This indicates that the fusion protein is produced after the induction.

For purifying the fusion protein thus obtained, the resulting culture solution is palletized by centrifugation, treated with lysozyme and ultrasonic waves and then centrifuged to obtain the inclusion bodies. The inclusion bodies are dissolved in 8M enzyme-20mM β-mercaptoethanol solution. The resulting solution is passed through a Sephacryl S 200 column and then the fraction from the protein peak portion is identified by SDS-PAGE. As a result, it can be seen that the obtained protein fraction contains mainly an about 45,000 dalton fusion protein (Figure 14).

The fusion protein thus obtained is added to 2M hydroxylamine-6M guanidine hydrochloride (6M GU HCl) , which is adjusted to pH 9.0 with lithium hydroxide, to a final concentration up to 5mg/ml and then completely dissolved therein. The resulting mixture is reacted at 45°C for 4 hours to induce the cleavage of the fusion protein into β- galactosidase and IGF-1. Then, the reaction is terminated by the addition of HCOOH to obtain a final pH of 2 to 3. The separated IGF-1 is identified with urea-SDS-PAGE. Figure 15 shows the result of urea-SDS-PAGE run where the IGF-1 produced is about 7,000 daltons.

The isolation of IGF-1 from the IGF-1/β-gal fusion protein hydrolyzed with hydroxylamine is conducted by using the property of IGF-1 being dissolved stably in a strong base. Specifically, the IGF-1 solution obtained from the cleavage with hydroxylamine is adjusted to pH 2 to 3 by adding small amounts of concentrated HCOOH and dialysed against 0.5M acetic acid (pH 2.88)-0.075M NaCl. The solution is loaded onto a SP-Sephadex C-25 ion exchange resin column. The column is then washed with dialysis buffer solution and adsorbed completely to the ion exchange resin. According to this, the majority of the β-galactosidase segments are removed as precipitates during dialysis, while a small portion of the β-galactosidase segments remains combined with the ion exchange resin. However, when the ion exchange resin is washed with the extraction solution (pH 5.0) containing 0.2M ammonium acetate and 0.2M NaCl, only the IGF-1 fragments are isolated from the ion exchange resin at pH 4.0 due to the difference of isoelectric points between the IGF-1 fragment and β-galactosidase fragment, and thus only IGF-1 is extracted (Figure 16).

The peak fractions identified in Figure 16 are collected and then dialyzed into the third-time distilled water. Then, the obtained fractions are identified with HPLC (Figure 17) and further examined by urea-SDS-PAGE (Figure 16). As a result, it is confirmed that the purity of the isolated IGF-1 is 95% or more with a molecular weight of about 7,000 daltons.

EXAMPLE 1

In order to obtain the DNA sequence encoding IGF-1, first, a cDNA library is prepared from liver tissue (Figure 2). In order to prepare the cDNA library, whole RNA is isolated from liver tissue utilizing the guanidine isothiocyanate method (Maniatis et al. 'Molecular Cloning', p. 194).

The whole RNA thus obtained is passed over an oligo (dT)-cellulose column to isolate only poly A^+ RNA (Maniatis et al. 'Molcular cloning', p. 197).

In order to check the purifying efficiency of the whole RNA and poly A RNA obtained above, it is confirmed whether all sizes of poly A RNA were obtained or not, by means of formaldehyde agarose gel. The poly A^+ RNA obtained above is used for preparing the cDNA library.

Thus, the isolated poly A^+ RNA is treated with oligo (dT)n primer and reverse transcriptase to prepare the corresponding cDNA. The cDNA-RNA hybrid is prepared and then hydrolyzed with RNase H. The ss-DNA thus prepared is converted into ds-DNA using Klenow fragment (DNA polymerase I) and the hairpin structure is removed by means of nuclease S1. The formed ds-cDNA is tailed with cytidine at the 3'-ends of each strand by using dCTP and terminal transferase. dsDNA wherein cytidine has been combined at the 3'-end of each strand is cleaved with the Pst I restriction enzyme. The Pst I fragment thus obtained is ligated with poly (dG)n tailed pBR 322 formed by using terminal transferase and dGTP (Maniatis, Molecular Cloning, p 215).

E. coli HB 101 is transformed with the above resulting vector to obtain the transformant containing many different dsDNAs. The group of cDNAs thus obtained is called a cDNA library (see:Figure 2).

EXAMPLE 2

Among about 200,000 transformants exhibiting tetracycline resistance, 80,000 colonies are examined using synthetic oligonucleotides to identify whether IGF-1 cDNA is included therein. The nucleotide sequences corresponding to five amino acids (Leu-Glu-Met-Tyr-Cys) present in from position 57 to position 61 of the IGF-1 amino acids sequence is synthesized (5'-CTG-GAG-ATG-TAT-TGC-3').

The 15-mer oligonucleotide thus synthesized is labelled at the 5'-position using P-ATP and T4 polynucleotide kinase. This labelled 15-mer oligonucleotide is purified using a 18% polyacrylamide gel before being used as a probe.

Using the probe labelled with ^{32}P at the 5'-end, the cDNA library is assayed for the presence of cDNA corresponding to the IGF-1 peptide.

Among the 80,000 colonies examined using colony hybridization method, 6 colonies were found to hybridize strongly to the probe. Figure 3 shows 2 colonies out of the 6 strongly hybridizing colonies. These positive colonies are rescreened to identify their ability to hybridize to the synthetic oligonucleotide.

One of these colonies includes a 680bp DNA sequence. In order to identify whether this DNA sequence correctly includes the cDNA sequence encoding IGF-1, this 680bp DNA sequence is digested with various restriction enzymes to determine its restriction map (figure 4).

From Figure 4, it can be seen that the 680bp DNA is inserted in Pst I cleavage site of pBR 322. In order to define this 680bp fragment more specifically, its sequence is determined. Figure 5 shows the 680bp sequence present in the Pst I cleaving site. In figure 5, the underlined portion indicates the sequence encoding mature IGF-1, and the sequence encoding for 14 amino acids at the 5'-end and 100 amino acids at the 3'-end and the 3'-noncoding region comprising 240 base pairs are present. In addition, after the 3'-noncoding region, the poly (A) sequence is present.

EXAMPLE 3

An attempt was made to express IGF-1 by using the cDNA sequence isolated according to example 2.

As mentioned above, 70 amino acids starting from glycine to alanine are expressed (see : Figure 6).

The expression vector pYJM-I4 which can express IGF-1 in E.coil. is constructed according to the following genetic engineering procedure.

IGF-1 cDNA is treated with the Ava II restriction enzyme and then weakly treated with the Alu I enzyme to separate an Ava II-Alu I DNA fragment of 206bp by gel electrophoresis. The obtained 206bp DNA fragment is mixed and ligated with four kinds of synthetic oligonucleotides, i.e. GATCCATTGGAGGATGAT-TAAATGG (MG-6), GTCCCATTTAATCATCCTCCAATG (MG-7), CTTAGTGAG (MG-8) and AATTCTCACTAAG (MG-9). This ligation product is inserted into the portion between the BamHI and EcoRI recognition sites of plasmid pUC 8 to prepare the plasmid pYJM-I1. The plasmid pYJM-I1 constructed above contains a cDNA sequence encoding 70 amino acids of IGF-1 between the BamHI and EcoRI recognition sites.

Thus, in order to express IGF-1 in the form of a fusion protein with β-galactosidase, said cDNA sequence is inserted into the β-galactosidase expression vector pCT 10 according to the following procedure to construct the expression vector pYJM-I4 which can express IGF-1 in E. Coli.

Plasmid pYJM-I1 is doubly treated with restriction enzymes BamHI and EcoRI to separate IGF-1 DNA, which is then filled in with Klenow fragment enzyme of E. Coli DNA polymerase at both ends, and then treated with Ava II restriction enzyme to separate 220 bp IGF-1 DNA fragment from the electrophoresis gel as can be seen from Figure 6. The obtained 220 bp fragment is mixed and ligated with two synthetic oligonucleotides, ATGATGATAAAG (MG-24) and GTCCTTTATCATCATCAT (MG-25). The ligated 235 bp DNA fragment is inserted into plasmid pCT 10 at Cla I site, which has been treated with Klenow enzyme, to prepare the plasmid pYJM-I4 (See : Figure 6). The constructed plasmid pYJM-I4 is identified by determining its DNA sequence (See : Figure 7).

As can be seen from Figure 6, plasmid pYJM-I4 comprises an antibiotic ampicillin-resistant gene (Ap^r), ColE1 replication point (ori.), and lac I gene (lac I) producing lac repressor. In addition, since in plasmid pYJM-I4, the DNA sequence capable of producing the fusion protein of β-gal and IGF-1 is linked to the tac promoter, which is a strong promoter of E. Coli, the pYJM-I4 plasmid can produce a large amount of β-gal / IGF-1 fusion protein. Further, since the DNA base sequence corresponding to $(Asp)_4$ -Lys,i.e., amino acid sequence, which is recognized by the enzyme enterokinase, is present in the linkage site of β-gal and IGF-1 protein, the expressed fusion protein can be treated with enterokinase to separate only IGF-1 (see : Figure 6).

EXAMPLE 4

The E. Coli strain JM 109 containing the expression vector pYJM-I4 is cultured overnight in LB + Ap (100 μg/ml) meduim to obtain a seed culture solution. 5ml of the seed culture is inoculated into 100ml of M9 + CA medium containing 100 g/ml of ampicillin and cultured at 37°C for 3 hours. To the resulting culture is added 2mM IPTG to induce the β-gal/IGF-1 DNA sequence linked to the E.coli tac promoter. Each 1.5ml of the thus obtained is collected and a portion of them is examined by SDS-PAGE. As a result, a strong band is observed at the position of molecular weight of about 50,000 daltons from each of the samples taken individually at 1,4 and 5 hours after the induction, in comparison with the absence of a band before the induction. From this result, it can be seen that the fusion protein of β-gal and IGF-1 is expressed under the control of tac promoter of E.coli (see Figure 9).

In addition, it can also be seen that in this procedure the protein is efficiently produced in an amount of above 20 to 30% of the entire protein of E. Coli. It can also be seen from the electron microscopic photograph that the produced fusion protein of IGF-1 and β-gal is accumulated as inclusion bodies in E. Coli (See : Figure 8).

Example 5

The isolation of IGF-1 of which the expression is induced in E. Coli using IPTG, starts by separating the inclusion body of β-gal / IGF-1 fusion protein formed in E. Coli.

For the separation of the fusion protein inclusion body, the cloned E. Coli strain cultured in the medium containing 2mM IPTG are collected by centrifugation, suspended in Tris-HCl buffer solution (pH 8.0) containing lysozyme, cultured at 37°C for 10 minutes, milled for 15 minutes using a ultrasonic mill, and then centrifuged at 1000xg for 15 minutes to collect only precipitates.

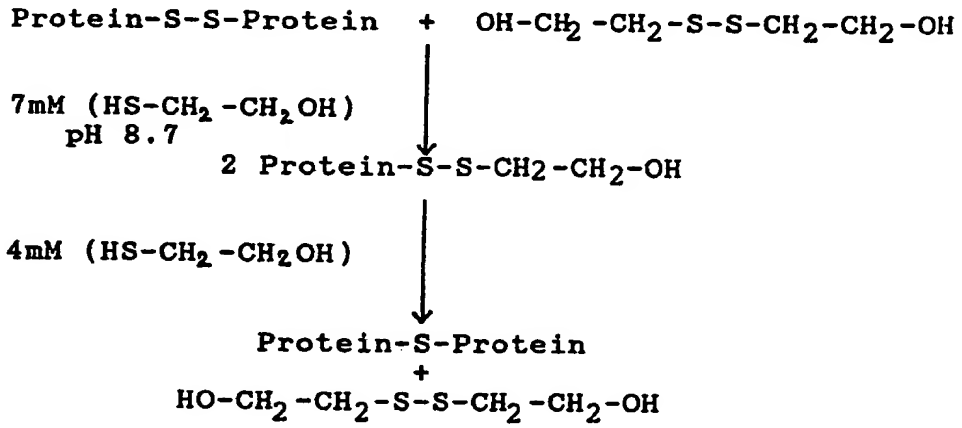
Since the β-gal / IGF-1 fusion protein inclusion body is not dissolved in an aqueous solution, the isolation of pure fusion protein from the separated β-gal / IGF-1 fusion protein inclusion body is carried out in the following manner. That is, the fusion protein inclusion body is dissolved in Tris-HCl solution containing 8M urea and 20mM β-mercaptoethanol. The resulting solution is centrifuged at 100,000xg for 90 minutes by means of a ultracentrifuge. Then the obtained supernatant is fractionated by passing it through a sephacryl S 200 column. This procedure is repeated two times to isolate the pure fusion protein. Each of the obtained fractions is identified by means of SDS-PAGE (See : Figure 10).

The identified β-gal / IGF-1 fusion protein fractions are introduced in a dialysis membrance and dialysed for 24 hours. After removal of urea and β-mercaptoethanol, the resulting fusion proteins are lyophilized.

In order to isolate only IGF-1 by treating the IGF-1 / β-gal fusion protein with enterokinase, the fusion protein must be dissolved in an enterokinase-active buffer solution (0.1M Tris-acetate buffer, pH 5.6). However, since the IGF-1 / β-gal fusion protein can be readily crystallized at low pH level due to disulfide bonds between protein peptides, the substitution of cystein is necessary in order to cleave the disulfide bonds. Such a substitution in the fusion protein is practiced by reacting the lyophilized fusion protein with 0.1M diethanol disulfide at 37 °C for 3 hours in the presence of 7mM β-mercaptoethanol. According to this method, the cysteins in the fusion protein are in the form of sulfhydryls allowing the fusion protein to be readily dissolved in an enterokinase-active buffer solution.

Enterokinase is added to the dissolved fusion protein and reacted at 20 °C for one hour to digest the linkage site between β-gal and IGF-1. As a result, the separation of β-galactosidase protein having molecular weight of 38,000 from IGF-1 protein having molecular weight of 7,000 dalton is occurred, as identified by SDS-PAGE (See : Figure 11).

For renaturation of the proteins, as can be seen from Figure 14, the isolated proteins are reacted in the presence of 4mM β-mercaptoethanol for 2 hours, and then, the concentration of β-mercaptoethanol is lowered by dialysis. According to this method, the disulfide bonds between protein peptides are induced and also the diethanol disulfide which has combined to the SH groups are removed.



EXAMPLE 6

In order to prepare the fusion protein from which IGF-1 can be separated by using hydroxylamine, as depicted in Figure 12, the BamHI-EcoRI fragment containing IGF-1 DNA is separated from plasmid pYJM-I1, treated with Klenow enzyme and then treated with Ava II to separate the 220 bp fragment which is then mixed and ligated with synthetic oligonucleotides, ATGTTA-ACG and GTCCGTTAACAT. Then, the ligated fragment is inserted into plasmid pCT 10 at Cla I site, which has been treated with Klenow enzyme, to prepare the expression vector pYPM-I1.

The fusion protein of β-galactosidase and IGF-1, which has been produced using this expression vector pYPM-I1 plasmid, contains asparagine-glycine residues from synthetic oligonucleotide located between β-galactosidase and IGF-1 encoding sequences. Thus, if the fusion protein is cleaved with hydroxylamine, the asparagine-glycine portion is cleaved and, therefore, only IGF-1 can be separated.

E. Coli strain JM 109 containing the plasmid pYPM-I1 is cultured according to the same manner as Example 4 and then the production of the fusion protein is induced by means of IPTG. Thereafter, SDS-PAGE confirms whether the fusion protein of β-galactosidase and IGF-1 is produced (See : Figure 13). The molecular weight of the produced fusion protein is about 45,000 daltons.

EXAMPLE 7

For isolation of IGF-1 produced by plasmid pYPM-I1, E. Coli strains containing plasmid pYPM-I1 are cultured according to the same manner as Example 4 and then the β-gal / IGF-1 fusion protein is partially purified according to the Method of Example 5 (See : Figure 14). This fusion protein fraction is dialysed to remove urea and β-mercaptoethanol, and then lyophilized. The lyophilized fusion protein is reacted with hydroxylamine at 45 °C for 4 hours to digest the dipeptide linkage between -galactosidase and IGF-1. The electrophoresis of the cleaved fusion protein shows an IGF-1 band at about 7,000 daltons, as can be seen from Figure 15.

In order to separate the IGF-1 and β-galactosidase produced by the cleavage with hydroxylamine, the solution containing a mixture of IGF-1 and β-galactosidase is adjusted to pH 2 to 3 by the addition of HCOOH and then dialysed in 0.5M acetic acid (pH 2.88) - 0.075M sodium chloride to induce the precipitation of β-galactosidase fragments while maintaining IGF-1 as dissolved in solution. The resulting mixture is centrifuged to remove the β-gal precipitate and the supernatant containing IGF-1 is adsorbed to a SP-Sephadex C-25 ion exchange resin column. Then, the adsorbed IGF-1 is extracted by washing the ion exchange resin with the eluent (pH 5.0) including 0.2M ammonium acetate and 0.2M sodium chloride. The light absorption of the extracted eluent is determined at 280mm UV light. The relevant peaks are identified with Urea SDS-PAGE, thereby screening and collecting only the desired IGF-1 fractions. In this case, the peak of IGF-1 fraction appears at pH 4.0 as shown in Figure 16.

The fractions corresponding to the peaks shown in Figure 16 are collected, lyophilized and then identified by urea-SDS-PAGE. As a result, a single IGF-1 band appears at the position of about 7,000 daltons (Figure 16).

HPLC of the isolated IGF-1 dissolved in distilled water identifies that IGF-1 is isolated in a pure state (Figure 17).

CLAIMS

1. A method for preparing IGF-1, wherein cDNA encoding IGF-1 is prepared from mRNA extracted and isolated from human hepatocytes and E. coli strain is transformed with an expression vector wherein said cDNA is inserted in the form of a fusion gene with another gene and then the resulting transformant is cultured in a suitable medium to produce IGF-1, which is characterized in that the said expression vector is a plasmid having enterokinase recognition site between IGF-1 gene and the other gene and that the resulting fusion protein is cleaved with enterokinase, followed by purification.

2. A method according to claim 1, wherein a vector having β-gal gene as the other gene and having DNA fragment encoding $(Asp)_4$-Lys as an enterokinase recognition site is used as an expression vector.

3. A plasmid pYJM-I1 (KCTC 8427P and KCTC 0004BP).

4. A plasmid pYJM-I4 (KCTC 8428P and KCTC 0005BP).

5. A method according to claim 1, wherein said expression vector is a plasmid pYJM-I4.

6. A method for separating a desired protein with enzyme, which comprises reacting a fusion protein with disulfide compound, which combines with the sulfhydryl group of cysteins present in the fusion protein but is not readily oxidized at normal temperature, in the presence of mercaptoethanol catalyst to prevent the formation of disulfide bonds between sulfhydryl groups present in the fusion protein and thereby exposing enzyme recognition site to the enzyme.

7. A method according to claim 6, wherein the disulfide compound is diethanol disulfide or dithiobisnitrobenzoic acid.

8. A method according to claim 1, wherein IGF-1 is separated by the method of claim 6.

9. A method according to claim 8, wherein IGF-1 having molecular weight of 7,000, which has been separated by cleaving β-gal / IGF-1 fusion protein with enterokinase is dissolved in 10mM Tris-HCl, and the resulting IGF-1 solution is introduced into the dialysis membrane and then gradually dialysed against 10mM Tris-HCl (pH 7.2) containing 4mM β-mercaptoethanol to remove the diethanol disulfide group combined with sulfhydryl group of cystein, thereby inducing the renaturation of IGF-1 and thus activate the protein.

10. A method for preparing IGF-1, wherein cDNA encoding IGF-1 is prepared from mRNA extracted and isolated from human hepatocytes and E. coli strain is transformed with an expression vector wherein said cDNA is inserted in the form of a fusion gene with another gene and then the resulting transformant is cultured in a suitable medium to produce IGF-1, which is characterized in that said expression vector is a vector having linker DNA containing AAC GGA codon which can be cleaved with hydroxylamine and that the resulting fusion protein is cleaved with hydroxylamine, followed by purification.

11. A plasmid pYPM-I1 (KCTC 8465P and KCTC 0006BP).

12. A method according to claim 10, wherein said expression vector is a plasmid pYPM-I1.

13. A method according to claim 10, wherein β-gal / IGF-1 fusion protein is produced by using synthetic linker encoding Asp-Val-Asn-Gly as a linker DNA containing AAC GGA codon which can be cleaved with hydroxylamine, followed by cleavage of the site between the Asn-Gly residues to obtain the mature IGF-1 having molecular weight of 7,000 and containing glycine as its first amino acid.

14. A method according to claim 10, which is characterized in that β-gal / IGF-1 fusion protein having Asn-Gly residues which can be cleaved with hydroxylamine between β-galactosidase and IGF-1, is partially purified and the resulting protein is reacted with 2M hydroxylamine-6M guanidine hydrochloride solution of which pH has been adjusted to pH 9.0 with LiOH, at 45 C for 4 hours to cleave the site between Asn-Gly residues present between β-galactosidase and IGF-1.

15. A method according to claim 10, wherein the β-gal/IGF-1 fusion protein is cleaved with hydroxylamine, the pH value of the solution is adjusted to 2 to 3 by adding HCOOH to stabilize IGF-1, the resulting solution is dialysed against 0.5M acetic acid (pH 2.88) - 0.075M NaCl to induce the β-galactosidase fragments to be precipitated and then removed by centrifugation, the supernatant solution containing IGF-1 is passed through SP-Sephadox C-25 column to allow IGF-1 to be adsorbed on it, the column is washed with 0.5M acetic acid (pH 2.88) - 0.075M NaCl and then IGF-1 is purified by extracting it from the eluent with 0.2M ammonium acetate -0.2M NaCl (pH 5.0).

16. A method according to claim 15, wherein the purified IGF-1 is further dialysed against distilled water and the obtained IGF-1 is subjected to HPLC using LKB ultrapac TSK OPS-120T 5 m column by means of a linear concentration gradient of 0 to 60 % of acetonitrile / redistilled water containing 0.1 % TFA (Tri-Fluoro Acetate).